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METHOD FOR PROMOTING AND CONTROLLING THE GROWTH AND FUNCTIONAL DIFFERENTIATION OF LIVING CELLS

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METHOD FOR PROMOTING AND CONTROLLING THE GROWTH AND FUNCTIONAL DIFFERENTIATION OF LIVING CELLS

[Seitari saibo no seicho oyobi ni kinodun ka]

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[There are no amendments to this patent.]

Claims

1. A method for promoting and controlling the growth and functional differentiation of living cells, characterized by the fact that living cells or living tissues selected from a group comprising connective tissue, nerve cells, glial cells, Schwann cells, skin cells, muscle cells, kidney cells, and liver cells are brought into contact with the surface of an artificial element that is provided such that multiple minute undulations are scored on said surface.

2. The method according to Claim 1, wherein the minute undulations are minute grooves.

3. The method according to Claim 2, wherein the minute grooves have dimensions of approximately 0.1-1000 mm wide and approximately 0.1-1000 mm deep.

4. The method according to Claim 3, wherein the minute grooves are parallel.

5. The method according to any of the aforementioned claims wherein the minute undulation surface is further coated with a biologically active substance.

6. The method according to Claim 5, wherein the biologically active substance is selected from a group comprising: collagen, poly-L-lysine, poly-L-orthinine, laminin, fibronectin, chick plasma, artificial lipid membrane (LB membrane, and the like), and nerve growth factor.

7. The method according to any of the aforementioned claims wherein the aforementioned artificial element is of at least one type of substance selected from the group comprising: quartz glass, hard glass, soft glass, organic high molecular materials, metal, ceramics, silicone rubber, and semiconductors.

Detailed explanation of the invention

[0001]

Industrial application field

The present invention relates to a method for bringing a surface, having a particular construction of artificial element, such as a cell culture vessel, artificial organ material, and similar artificial element into contact with living cells or living tissues, causing these cells or tissues to express affinity or decreased protective reaction, and promoting and controlling growth and functional differentiation, and it is useful for cellular engineering, tissue culture, medicine, and artificial organs as well as their related cross-disciplinary fields.

[0002]

Conventionally, there have been 2 different kinds of technical improvements that have advanced cell-culture technology in controlled artificial environments for multiplication, differentiation, growth, and the like of animal cells, plant cells, bacterial cells, fungal cells, etc..

Namely, improvements in the culture vessels that come into direct contact with the cells, and improvements in the media that offer nutrients to the cells. Of these, the main approach in the past has been in the latter.

[0003]

In Japanese Kokai Patent Application 60[1985]-18174, a method was proposed for inducing new bones by filling the bone defect area with a porous substance such as a ceramic sintered body, for example, as a prosthetic material, and utilizing the porous body for its effect as a biofilter against collagen fibers and osteoclastic cells. Nevertheless, this method is a preventive method for inducing new bones by preventing the invasion of substances that inhibit bone growth, and does not offer an effect such as controlling the alignment of growth, or positively increasing or suppressing growth rates by improving the surface condition of the physical body that comes into direct contact with the living cells or living tissues.

[0004]

On the other hand, Japanese Kokai Patent Application Sho 61[1986]-176339 proposes a blade-shaped intraosseous implant in which a staggered-stage intersecting structure is formed by connecting multiple through-holes in the part embedded in the bone. The object of this proposal is to increase the mechanical binding power in order to prevent the implant from dropping out or wobbling. It is not designed to express an effect such as controlling the growth alignment, or inhibiting or increasing the growth rate of living cells or living tissues, as was the case in the aforementioned proposal.

[0005]

Also conventionally, with regard to artificial organs and other therapeutic hardware designed for long-term *in vivo* implantation, the main effort in improving materials or macroscopic shapes has been focused on heightening the affinity or reactivity with connective tissue cells, thus reducing the so-called defense reaction.

[0006]

Nevertheless, although there is great diversity in the macroscopic shapes (plate-like, dish-like, tubular, etc.) and materials used in the aforementioned culture vessels or artificial organs presently in use, the surfaces of these vessels and hardware, [namely] the shape of the part that comes into direct contact with cells and that directly relates to cell growth and the like, is left alone without much design being devoted to it, so that the surface is either the even surface shape intrinsic to the material, or [if intrinsically uneven] it is subjected to surface finishing to make it

smooth. We still have not seen any examples proposed of improvements or research focused on the fine structure of the surface.

[0007]

Furthermore, with regard to technology for controlling the growth alignment of living cells or tissues, conventionally as a technology for orienting the growth of neurites, for example, there has been [the technique] of causing oriented growth at sites where chemical substances such as fibronectin, laminin, collagen, polyornithine, NGF (nerve growth factor), and the like are present or along these sites. In this method, these chemical substances must be disposed by giving each individual molecule an orientation, accordingly an extremely high technological level is required, and these methods have the shortcomings in stability, in which specific properties are lost when chemical substance becomes inactive.

[0008]

Also, as one of the known conventional methods, there is growth induction by electric field orientation where living tissues and cells are oriented along an electric field, but this method has problems such as, for example, the fact that effect of electric fields on living tissues is not sufficiently understood.

[0009]

Problems to be solved by the invention

As a result of explicating the microstructures on the surfaces of artificial elements and their special nature and behavior on the living cells and tissues with which they come into contact, which the prior art had viewed casually, the inventors succeeded in solving the problems described above, thus arriving at the present invention.

[0010]

The first object of the invention is to offer an artificial element, for example medical hardware, a culturing vessel for cells or tissues, and the like, furnished with a surface to which living cells and tissues that come into contact will show enhanced affinity and decreased host defense reaction.

[0011]

Another object of the invention is to use an artificial element of a special construction that will make it possible to control neuranagenesis, to control cell growth, and to control cell multiplication, with stability and persistence of effect.

[0012]

Means to solve the problems

The aforementioned object is accomplished by a method for promoting and controlling the growth and functional differentiation of living cells, characterized by the fact, that living cells or living tissues selected from a group comprising connective tissue, nerve cells, glial cells, Schwann cells, skin cells, muscle cells, kidney cells, and liver cells are brought into contact with the surface of an artificial element that is provided such that multiple minute undulations are scored on said surface.

[0013]

More specifically, according to the invention minute undulations are scored by mechanical or chemical means on a surface; being the surface of an artificial element such as a cell culture vessel or therapeutic hardware that comes into contact with living cells or living tissues selected from a group comprising connective tissue, nerve cells, glial cells, Schwann cells, skin cells, muscle cells, kidney cells, and liver cells thus making it possible to increase adhesion and selectivity for cells and tissues, and to control cell multiplication. And by making these undulations grooves, it is possible to form groups of cells and grow cells oriented along the grooves.

[0014]

Operation

The following statements explain the mechanism of the invention and its operation in greater detail. The invented method is applied to the surface portion of the artificial element that comes into contact with living cells and tissue, which is given a rough pore surface by minute undulations or more preferably fine grooves. These fine grooves may have a depth and width ranging from approximately 0.1 mm through 1000 mm, and need not necessarily be parallel, and the depth and width need not be homogeneous and need not be a regular shape. More specifically the depth and width of the fine grooves may vary appropriately within the aforementioned ranges according to the materials and shape of the artificial element. The cross-sectional shape of the grooves may be selected from any shape such as V-shape, U-shape, or dovetail shape, for example. The planar shape may be any shape such as a straight line, curved line, or waveform, and the aforementioned specific effect will be exhibited even if a complex fine surface structure is made with these types overlapping each other.

[0015]

Nevertheless, the best results are obtained when the shape and array of the aforementioned fine grooves are straight-line fine grooves parallel to each other whose width and depth are within the aforementioned range, for not only does this make it possible to control cell multiplication, to control cell growth, and to control neuranagenesis, but it also ensures the astounding effect that the living cells or tissues grow easily and reliably in the desired direction, with good conformance. In other words, the property of growth with the connective tissue cells in the middle of the grooves, and conversely the nerve cells and the like on top of the ridges is conspicuously expressed, thus accomplishing oriented growth along the grooves.

[0016]

Furthermore it is possible to further enhance [the effect] by coating the surface undulations of the artificial element having the aforementioned properties with a biologically active substance, preferably a substance selected from the group comprising collagen, poly-L-lysine, poly-L-orthinine, laminin, fibronectin, chick plasma, LB factor (artificial lipid membrane), and NGF (nerve growth factor).

[0017]

There are no particular limitations on the material for the artificial element that is to be scored with the aforementioned minute undulations, but ordinarily it will be at least one type of substance selected from the group comprising quartz glass; hard glass; soft glass; organic high molecular materials such as polystyrene or polyvinyl chloride and other plastics, collagen, cellulose, agar, and the like; metal; ceramics such as SiN, BN, or apatite, for example; silicone rubber; and semiconductors such as Si, Ge, GeAs, InP, GaSe, or InSe, for example; or it may also contain a surface, for example, of collagen plate or plasma clot.

[0017] (sic; 0018)

There are no particular limitations on the overall macroscopic shape of the artificial element, for example vessel or hardware, to which the present invention is applied. More specifically, it may have any shape according to its object and application, whether plate-shaped, dish-shaped, spherical, fibrous, tubular, or granular, for example, as long as it is possible to provide the aforementioned minute undulations on the surface of those parts that come into contact with living cells and tissues. Generally, artificial elements that are suitable for cell culturing vessels or therapeutic hardware such as artificial organs, for example, are flat plates, circular disks, fibers or cylinders of thickness from 10 mm to 10 cm, spheres of diameter of 100 mm through 10 cm, or hollow cylinders with an outside diameter of 10 mm through 10 cm, for example.

[0019]

A surface provided with such minute undulations exhibits the special effect of cell multiplication properties and cell adhesion properties not observed in prior art unfinished surfaces, and adds to the control of cell alignment and the like. These effects will differ depending on the differences between the aforementioned material for the element, and differences will also be observed according to the element's macroscopic shape. There will also be differences depending on the type of cell or tissue confronting the element, and depending on the species, sex, age, and other factors of the animal from which the cells or tissues are derived. Nevertheless, the special properties arising from the fine undulating structure of the surface, which is the effect of the element, universally and obviously exceed the effects of the aforementioned factors, and enable the object of the invention to be sufficiently accomplished.

[0020]

Figure 1 is an enlarged oblique drawing schematically showing the growth of cells on a quartz glass plate that has been subjected to minute groove finishing.

[0021]

In this figure, several straight minute grooves 2 of cross-sectional U-shape have been scored on quartz glass plate 1. As living cells that are brought into contact with this surface and cultured, there are the nerve cells NC on the surface part of ridge 3 between the minute grooves, which reproduce nerve axons AX along the ridges, whereas connective tissue cells GC such as glial cells and the like grow processes along the groove in groove 2.

[0022]

Figure 2 shows an illustration of the circumstances of growth of cell C along groove 2 that has been similarly scored on glass plate 1. Figure 3 shows the condition of cell C growth along groove 2 scored on the surface of plastic fiber 1' parallel to the fiber axis.

[0023]

The scoring of minute undulations on the artificial element used in the invention method may be by appropriate application of the photoresist method, replica method, scratch method, press method, or etching method and the like. For example, lithographic techniques may be applied to finishing the surface of a glass dish or glass plate and the like, as shown in Figure 2. Or the identical method may be applied to fine finishing of the fiber surface shown in Figure 3, or to the surface of a glass sphere or the inner surface of a glass tube. Three main methods may be used to finish plastic plates, fibers, and the like. Namely, (1) the aforementioned lithographic technique,

(2) the method of taking a replica of a glass plate finished by lithography, and (3) the method of making scratch grooves on the surface by a cutting plane of fibers or minute particles. The glass tube replica method or the scratch method are good for the fine finishing of surfaces of silicone rubber, plastic tubes, and similar surfaces or surfaces of fibers, plates, or tubes and the like using collagen as the material.

[0024]

Figure 4 explains the gist of the method for transferring a fine structure by the replica method to plastic or silicone rubber by offering a practical implementation where replica 5 is an artificial element of silicone rubber or the like, in which prototype 4 of a material such as metal or quartz glass plate on which the minute grooved structure has been scored is used to transfer the fine grooved structure to substrate 1.

[0025]

Figure 5 shows an example of the method for scoringly establishing a fine groove on the surface of a fibrous material. Here fiber 1' is obtained as the artificial element with minute groove 2 scored on its surface by drawing a fiber in the direction of the arrow through the inside of prototype 4', being a metal or glass cylinder having fine grooves scored axially on the inner wall.

[0026]

Figure 6 shows a classical implementation of the scratch method. Here minute grooves are scored by scratchlike scarring of a plate surface by causing a dentate or serrate prototype 4" composed of a hard material to come in contact with the surface of plate 1 composed of a softer material such as plastic, rubber, or similar substance such that prototype 4" and plate 1 move relative to each other along the direction of the arrow.

[0027]

Practical examples

The following statements further explain the invention by means of practical examples.

Practical Example 1

Dorsal spinal root ganglion cells were taken from an adult mouse and treated with enzymes such as trypsin and collagenase, for example in order to isolate cells. These cells were then cultured respectively on quartz glass on which minute grooves had been scored at a width of 0.5 mm and depth of 0.2 mm, and a smoothly surfaced quartz glass having no minute grooves. The culture broth was a 1:1 mixture of Hams F-12 medium and Dulbecco's MEM to which

progesterone, insulin, and transferrin were appropriately added. The culture was conducted for 24-48 h under serum-free conditions at 37°C in air containing 5% carbon dioxide. The results of culturing and the condition of nerve process regeneration on the glass plates are shown in Figure 7 and Figure 8.

[0028]

Figure 8 shows the regeneration of nerve processes on quartz glass that has not been furnished with minute grooves; axon regeneration has occurred but without fixed orientation. On the other hand, when the [cells are grown] on quartz glass furnished with minute grooves as shown in Figure 7 (because they have a depth of 0.2 mm and width of 0.5 mm, which is beyond the resolving limit of visible light, the minute grooves cannot be observed), axon regeneration is observed oriented along the direction of the minute grooves. In this case processes that have departed from the grooves can be observed, but their length does not exceed 20% at the most of the projections oriented to the grooves.

[0029]

Results identical to the above were observed, with minor differences, when the identical method used above was used to culture cells, varying the type of nerve cells, animal species, age, and perineural tissue, and using elements composed of materials such as soda glass, plastic, collagen, and apatite, for example, that were respectively furnished with minute grooves.

[0030]

Practical Example 2

Dorsal spinal root ganglion cells and spinal motor nerve cells were separately taken from chick fetuses 15 days after fertilization and cultured simultaneously on both ends of grooves on a plastic plate finished with grooves 5 mm wide and 2 mm deep. This resulted in growth of the dorsal spinal root ganglion cells and motor nerve cells along the grooves, with high efficiency of synaptic couplings between them. At this time, electrical stimulus applied to the dorsal spinal root ganglion cells was also detected in the motor nerve cells, thus obtaining the characteristic threshold value characteristics in synaptic couplings. (Enhancement of regeneration of nerve fibers and nerve cells)

[0031]

Practical Example 3

Mature mouse dorsal spinal root ganglion cells were cultivated according to the method of Practical Example 1 on elements to which the present invention had been applied, with the

material being glass or plastic having grooves 2-10 mm wide and 0.5-1 mm deep on the surface. However in this example fetal calf serum (FCS) was added to the medium and it was cultured for 84 h. The dorsal spinal root ganglia contain nerve cells and additionally Schwann cells and glial cells, and these cells multiplied in the medium. On the invented element nerves grew 90% or more in the top grooves, and conversely the non-nerve cells grew 90% or more below the top grooves. From this difference in properties, the nerve cells may be readily separated from other cells, so they can be respectively separated and harvested. (Cell sorter function)

[0032]

Practical Example 4

Fibroblasts, Schwann cells, skin cells, skeletal muscle cells, kidney cells, and liver cells obtained from chicks on the fifteen day after fertilization were cultured on elements finished by the application of the invented method. They showed specific cell multiplication and cellular arrangement, and it was clear that there was a natural relationship between the element shape and cell selectivity and cell growth stimulus. Above all, a pronounced effect was observed in culturing cells and tissues derived from organs with particular cellular arrangement. These particulars varied greatly depending on the age and species of animal cell donor, and the culturing method. For each respective cell there was an optimal element material, groove shape, width, depth, and other parameters. (Selectivity for different types of cells)

[0033]

Practical Example 5

Fine grooves 10 mm wide and 3 mm deep were scored over a certain range on a quartz glass plate, and the rest of the surface was allowed to remain smooth. On the glass plate cells were cultured identical to the method described above for Practical Example 1. As a result, growth of nerve cells and connective tissue occurred along the grooves in the grooved portion 6, as shown in Figure 9, but growth in the ungrooved portion 7 was irregular.

[0034]

Practical Example 6

The quartz glass plate of the aforementioned Practical Example 5 was again used, and a single type of cells (connective tissue cells) that did not include nerve cells was cultured according to the identical method used in that practical example. As shown in Figure 10, there was a pronounced difference in cell growth orientation between grooved portion 6 and ungrooved portion 7, and there was also a difference in cell adhesion and cell selectivity and similar parameters.

[0035]

Effect of the Invention

In the invention, a minute undulating structure is mechanically or chemically scored on the surface of a cell or tissue vessel or therapeutic hardware that comes into contact with living tissue or living cells selected from a group comprising connective tissue, nerve cells, glial cells, Schwann cells, skin cells, muscle cells, kidney cells, and liver cells, which makes it possible to increase adhesion and selectivity for cells and tissues and to control cell multiplication; and when these undulations are fine grooves, or particularly if they are parallel straight fine grooves, it becomes possible to cause oriented growth of cells and tissues along the grooves, thus making it possible to have elements of even better quality for culture vessels or therapeutic hardware.

[0036]

In other words, the effects of the method of the invention may be summarized as follows:

1. Effect in promoting cell growth

1-A. Effect of promoting cell division and accelerating cell multiplication.

When cells are cultured according to the invented method, the time required for cell division is shortened, and the number of cells grown within a unit of time (for example 1 day) is increased by a factor of 2 or more. At this time various phenomena associated with cell division are observed, such as simultaneous increase in the cell's nuclear DNA and the amount of protein synthesized. These effects are pronounced in cells with excellent cell division potential, namely liver cells, kidney cells, skin cells, and angiogenic cells.

[0037]

1-B. Effect in promoting cell growth

An effect is observed wherein cells without division potential or with low division potential grow larger, or the fibers growing from the cells are longer. Specifically, nerve cell fibers grow 2-5 times longer, skeletal muscle fibers grow 2-3 times longer, and the diameter is 2-4 times greater.

[0038]

2. Effect in promoting cell function differentiation

Within a living system, cells not only perform their roles solo but they also perform roles in coordination with neighboring cells, with a division of functions. The element used in the invented

method is observed to allow expression of higher functions that have not been observed in conventional elements.

[0039]

A. When liver cells or kidney cells are cultured according to the invented method, not only does the number of cells increase, but the expression of higher cellular functions is promoted.

[0040]

More specifically, in liver cells the activity of enzymes directly involved with detoxification, such as beta-glucuronidase, increases by a factor of 5 or more. Such an increase in specific enzyme activity has not been realized in conventional culture environments.

[0041]

In kidney cells, an increase was observed in the activity of several enzymes directly related to cellular urogenous and excretory functions.

[0042]

In skin cells, various phenomena were observed that are thought to promote formation of a more differentiated skin structure, such as promotion of basal membrane protein synthesis, or promotion of the synthesis of keratin proteins that are thought to depend on a multilayered cellular arrangement.

[0043]

B. Even in nerve cells and muscle cells where cell division rarely occurs, functional differentiation is promoted, and higher functions are exhibited.

[0044]

In nerve cells, not only is the growth of nerve fibers given an orientation as described above, but also functions characteristic of nerve cells, namely functions that accompany the formation of neural networks, are expressed. More specifically, in nerve cells there is an increase in the activity of enzymes for synthesizing chemical transmitters, for example choline acetylase and catecholamine-synthesizing enzymes. There was also a several-fold increase in the release of chemical transmitters into the culture broth. An increase was also observed in various enzymes in the nerve cells exhibiting heightened nerve function.

[0045]

In muscle cells, not only was there an increase in their length and diameter, but there was also an increase in activity of creatine phosphokinase, which indicates an increase in muscle contractile function.

[0046]

As summarized above, (1) when the invented element was used, phenomena were observed that were mostly unobserved when conventional cell culturing techniques were used; (2) these phenomena are considered indicative of the extreme differentiation of cell functions and indicative of a condition that approximates their working within a living system.

[0047]

The element used in the invented method is not merely finished by conventionally known methods, specifically simply coating a growth-inducing chemical substance on the surface of a culture vessel or medical hardware and the like, or by heat or electric discharge, but has been subjected to mechanical or chemical finishing of specific minute undulations, and a groove structure above all, on the surface of the element, so not only is the finishing technology relatively easy and simple and can be applied to mass production, but it also maintains a stable effect and characteristics over a long period of time.

[0048]

Furthermore, as one example of a conventional method, it is known that when an electric field is involved the growth of living tissues or cells can be induced oriented to the electric field; but this method has problems, for example in that the influence of electric fields on living tissues is not sufficiently understood. An element to which the present invention has been applied can be used as an element for helping to elucidate these problems, and this quality also allows it to be used as a cell sorter.

[0049]

Above all, this element is particularly excellent in controlling cell alignment and cell selectivity, and can be [used] as an element for medical hardware placed in an organ or apparatus that requires a specific cell alignment. More specifically, conventionally medical hardware intended for long-term implantation in living systems aimed at reducing the so-called defense reaction of the living system, and the main effort for improvements in materials and shapes was devoted to this goal. However the element with which the invention is concerned is subjected to a surface finishing method, so it has specific concordance to specific cell groups, and has specific

cell selectivity that controls the alignment of cells on the element's surface, therefore this property is utilized so it can be used as an element for covering the surface of various types of medical hardware. Above all, its has high reactivity with and affinity for connective tissue cells, so by skillfully designing the material for the element and the finishing for the grooves, it can be applied as medical hardware having the property of low host defense reaction.

[0050]

As explained above, the present invention does not have the problems of concern when chemical substances or electric fields are used, and has the advantage of easily and assuredly enabling the growth of living cells and tissues in the desired orientation. Accordingly, the invention offers a new method for biotechnology such as the formation of specific synapses, for therapy for promoting healing of injuries and the like, and for extraction of substances from cells, and it can be applied to the production of substances.

[0051]

What we particularly want to emphasize is that the invention is not limited to culture vessel materials, size, shape, or other parameters, and can be used as an add-on technology supplementing the prior art without losing any of the improvements advanced by the prior art in the materials or shape of hardware, and without changing their macroscopic shape. The present invention may be thought of as increasing the performance of existing culture vessels and medical hardware by applying fine surface finishing to them, giving them cell specificity and cell growth control properties that they did not previously have. Furthermore, there are not very many stipulations about the original material of the element to which the invention applies, and it may be applied to vessels and hardware constituted of one type or multiple types of materials; there is a high possibility that new vessels and medical hardware will be produced having properties not considered by the prior art; it may be called an unprecedented invention with the following applications being anticipated for the future.

[0052]

(1) Artificial organs and medical hardware placed in living systems. Above all as a surface element for artificial blood vessels, artificial hearts, and pacemakers. Also, as an element for nerve sutures and materials for transplantation.

(2) Biochips and biocomputers.

(3) Cell isolation devices (cell sorters) and cell cloning devices.

(4) Hardware for organ transplantation, brain, nerve transplantation.

Brief description of the figures

Figure 1 is an enlarged oblique view explaining the state where living cells are growing on an artificial element according to the invented method.

Figure 2 is a plan view explaining the state where living cells are growing on an artificial element according to the invented method.

Figure 3 is an oblique view explaining the state where living cells are growing on an artificial element according to the invented method.

Figure 4 is a schematic side view explaining the method for preparing an artificial element suitable for use with the invented method.

Figure 5 is an oblique view explaining the preparation of another concrete embodiment of an artificial element suitable for use with the invented method.

Figure 6 is an oblique view explaining the method for preparing yet another concrete embodiment of an artificial element suitable for use with the invented method.

Figure 7 is a sketch of a microphotograph showing the condition in which nerve projections have been regenerated on an artificial element suitable for use with the invented method.

Figure 8 is a sketch of a microphotograph showing the condition in which nerve projections have been regenerated on a conventional, publicly known artificial element.

Figure 9 is a sketch of a microphotograph showing the effect of the invented method.

Figure 10 is a sketch of a microphotograph showing the effect of the invented method.

1 Glass plate
1' Plastic fiber
2 Fine groove
3 Ridge
4 Prototype
4' Prototype
4" Prototype
5 Replica

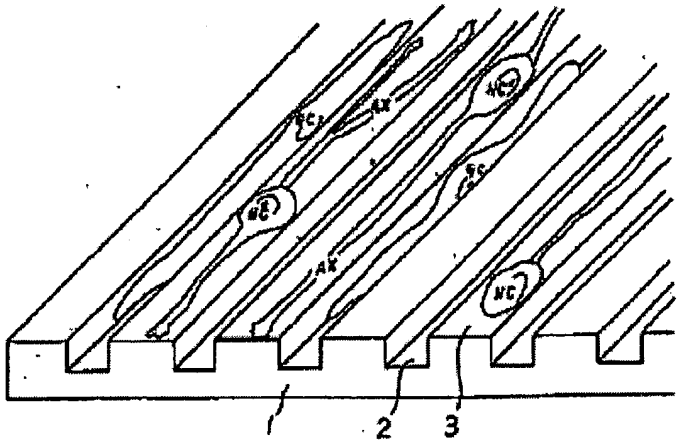

Figure 1

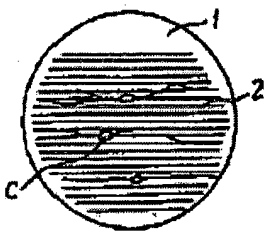

Figure 2

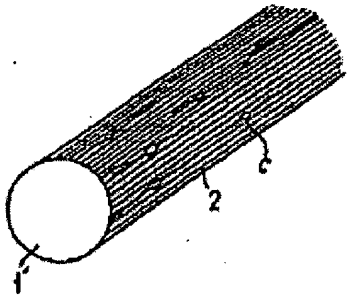

Figure 3

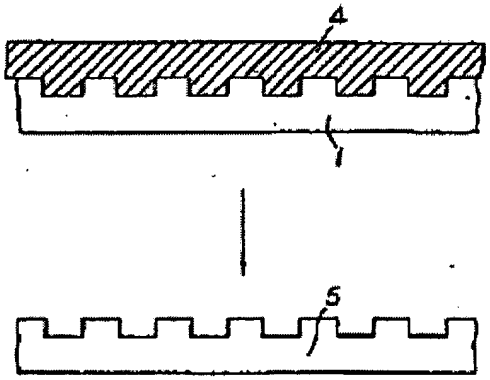

Figure 4

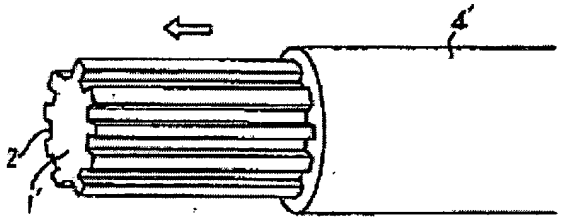

Figure 5

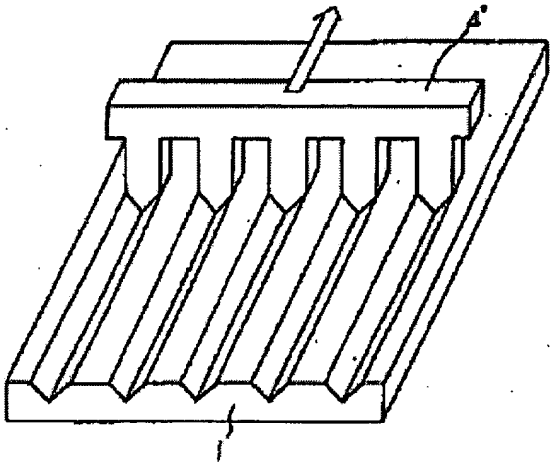

Figure 6

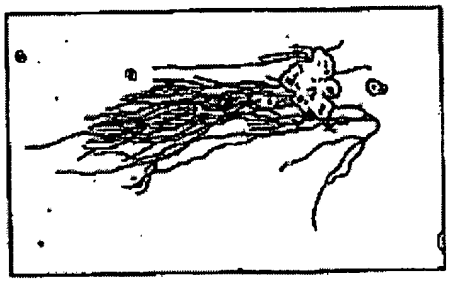

Figure 7

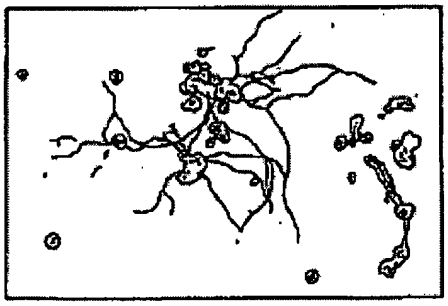

Figure 8

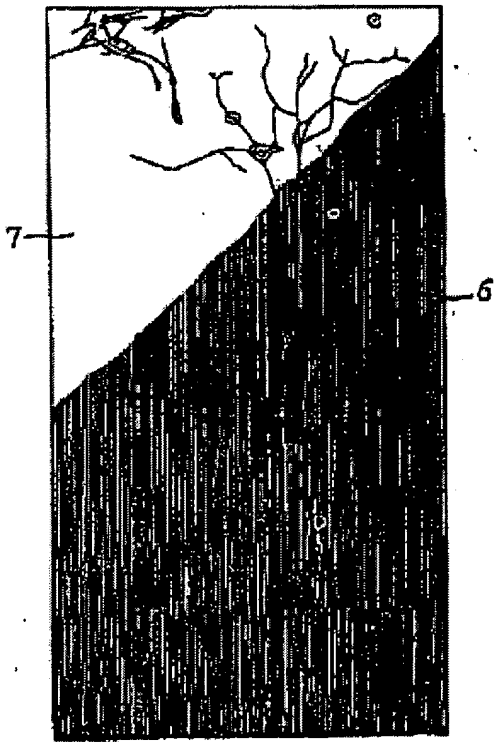

Figure 9

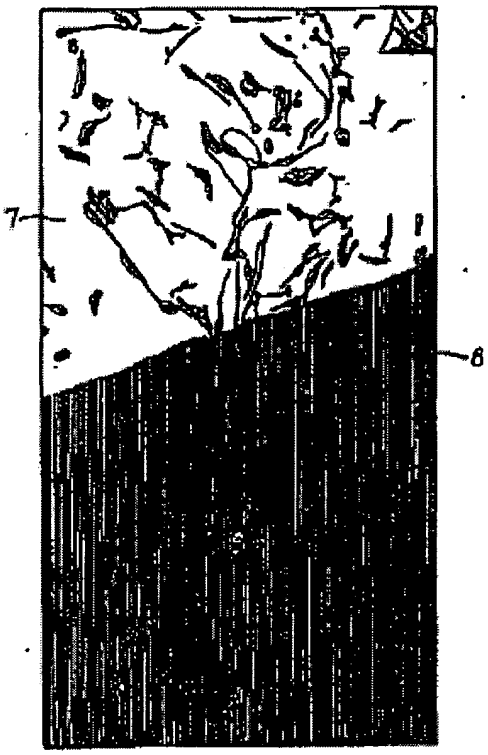

Figure 10